BADGES OF IMMOBILIZED ENZYMES: DETECTION OF CHEMICAL WARFARE AGENTS

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ABSTRACT

Rapid detection of chemical warfare agents (CWAs) in all of its forms including gas and liquid is of paramount importance to protect the warfighter or first responders in homeland defense. CWAs such as OPs and vesicants pose serious threats because these agents can be ingested with contaminated food or rapidly penetrate skin. We reported that polyurethane immobilized cholinesterases (acetyl and butyryl) yielded a product with similar enzyme activity as soluble enzyme. While enzyme sensors have the advantage of selectivity, sensitivity and, most important, specificity, ChEs are particularly ideal enzymes for rapid detection of OPs because they possess some of the fastest turnover rates known for any enzyme, and are the most sensitive targets for CWAs including GA (tabun), GB (sarin, the agent used by the terrorist group in Japan in 1995), GD (soman), and VX. We also reported that immobilized cholinesterase biosensors exhibited remarkable stability suitable for detection of OPs in both air and water, and stability to harsh environmental conditions because the immobilized enzymes do not leach from the polyurethane support. We now describe two improvements over current OP detecting kits. (1) Immobilized enzyme badges are being designed as a field system capable of identifying the type of OP present. This will aid in treatment. (2) A coupled enzyme reaction also provides rapid colorimetric or electrochemical indication of vesicants (HD). Therefore, one badge detects two classes of CWAs. With the constant threat of chemical warfare or terrorist acts, the development of alternative means to rapidly identify CWAs is critical.

INTRODUCTION

Enzyme sensors have the advantage of selectivity, sensitivity and, most important, specificity, ease and portability, and markedly simplified instrumentation. Biosensors based on cholinesterases (ChEs) immobilized non-covalently have been prepared by a variety of processes. The currently fielded spot M256A1 chemical agent detector kit and the M272 water test kit use dry eel ChE non-covalently applied onto fiber or ion-exchange paper. It can only be exposed to air/vapor environmental conditions. An immobilized enzyme will not leach from the polyurethane support so that the product - an OP badge - can now be used to sample anything from soil, water, to air.

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It has been demonstrated that a variety of enzymes exhibited enhanced mechanical and chemical stability when immobilized on solid supports, thus producing a biocatalyst. The study of degradation of organophosphates by immobilized enzymes dates back to Munnecke, who attempted to immobilize a pesticide detoxification extract from bacteria by absorption on glass beads. The absorbed extract retained activity for a full day. Wood and coworkers, using isocyanate-based polyurethane foams (Hypol®), found that a number of different enzymes could be covalently bound to this polymer and retain their activity; after that Havens and Rase successfully immobilized parathion hydrolase.

METHODS

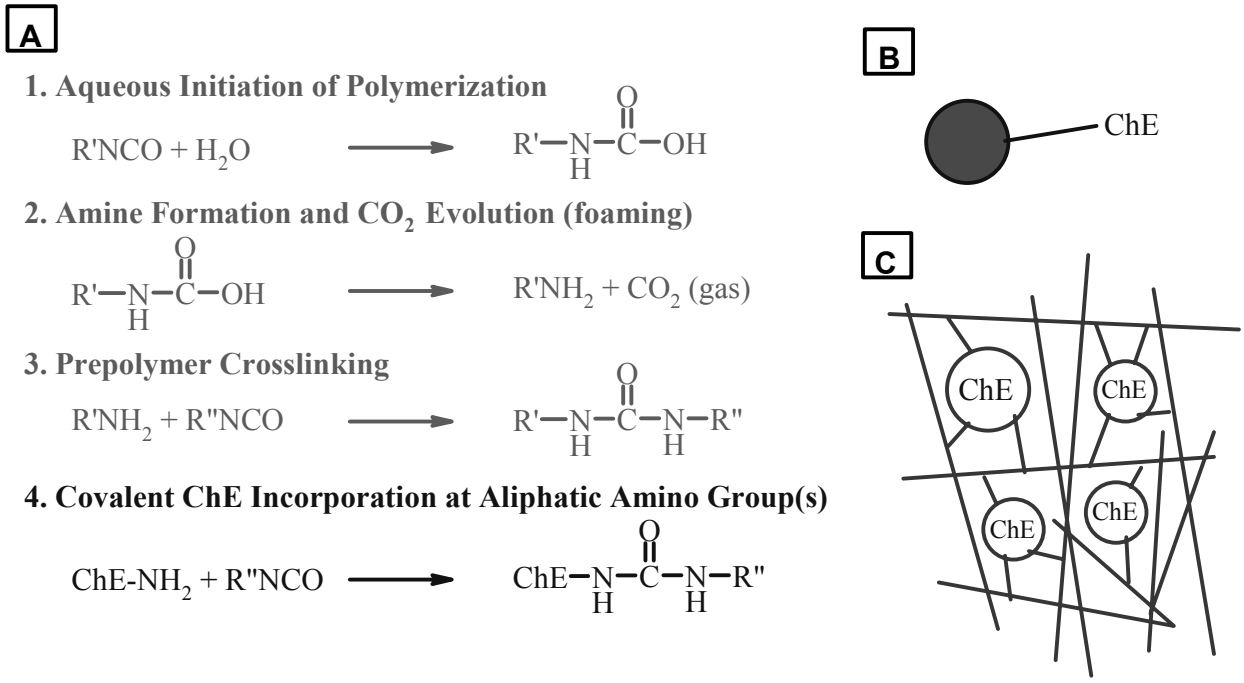

Figure 1. Scheme for Crosslinking ChEs to TDI Polyurethane.

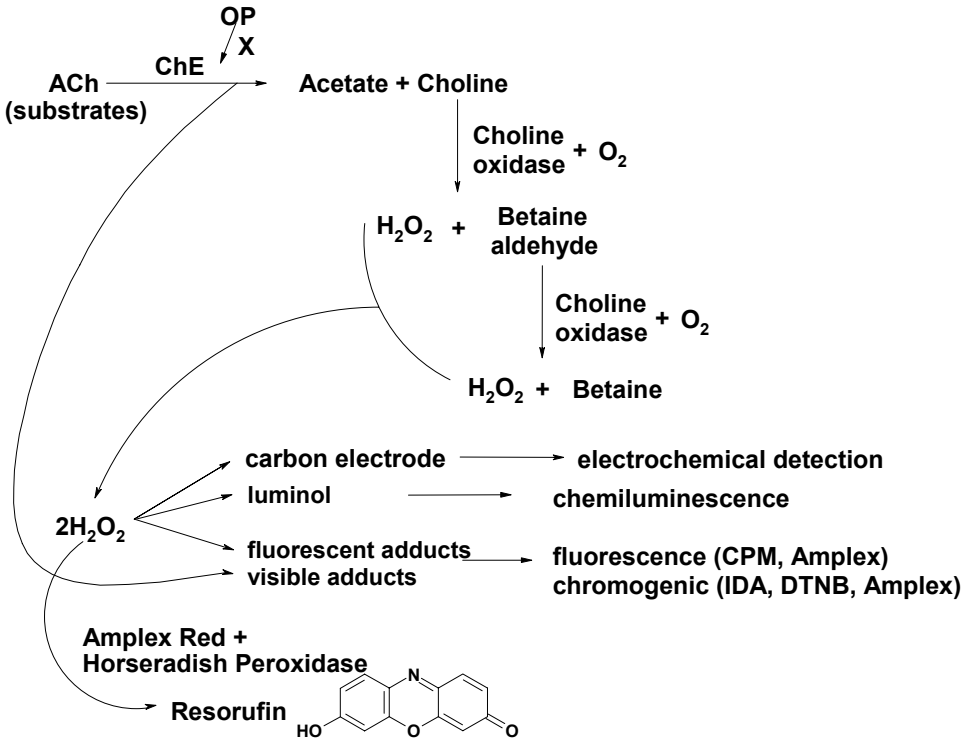

Figure 2. Schemes for Detecting Biosensor Activity.

Sponge synthesis and assay: The immobilized enzymes can be synthesized and cured in less than 20 minutes at ambient temperature and molded into the shape of any container; a new technique was utilized to mix the prepolymer (Hypol prepolymer TDI 3000, Hampshire Chemical, Lexington, MA) and enzyme in buffer containing 1% surfactant (Pluronic P-65, BASF Specialty Chemical, Parsippany, NJ) (Gordon et al., 1999). As shown in figure 1, (A) Reactions 1-4 show the interaction of the prepolymer with water and free amino groups on the surface of cholinesterases (ChEs) or any protein. (B) Classical covalent linkage to a preformed solid support is shown in the upper right, where the enzyme is distant from the support. (C) In contrast, the result of *in situ* polymerization with enzyme is depicted in the lower right, where the enzyme becomes cross-linked to and a part of the matrix during synthesis. Computer generated models of the surface moieties of ChEs that are available for cross-linking can be observed as one rotates the enzymes, and do not interfere with the active site. In this manner, the enzyme gains some of the structural integrity of the cross-linked polymer. In contrast, the model of laccase based on the crystal structure depicts a lysine in the active site, indicating that the immobilization process interferes with resulting enzyme activity. Furthermore, this enzyme is immobilized with the lowest efficiency. For our purposes, the sponge containing the immobilized enzymes were molded in a Tupperware® container, and then cut as desired. Alternatively, the immobilized product can be spotted (as a dot of glue) onto a paper or rigid plastic backing, to make a biosensor.

Activity: Several different techniques to determine the activity of immobilized enzymes are shown in the biosensor detection scheme (figure 2. Detection can be performed qualitatively by the human eye for visible chromogens, or dark-adapted eyes for chemiluminescent chromogens, or quantitatively using portable handheld devices, which measure fluorescence, chemiluminescence and visible chromogens. As shown in figure 3, the coupled enzyme reaction, acetylcholinesterase followed by choline oxidase to yield hydrogen peroxide, can be optimized to the same pH.

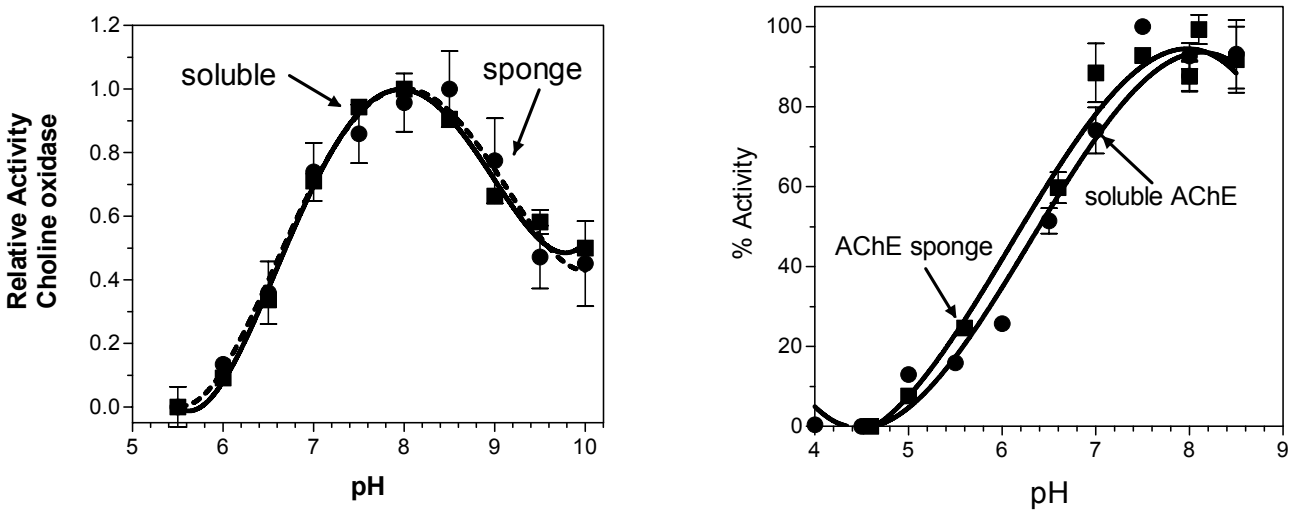

Figure 3. Both choline oxidase (*left*) and acetylcholinesterase (*right*) have the same pH optimum, permitting optimization of the coupled reaction illustrated in figure 2.

We found that the TDI sponge has a significantly higher loading capacity for ChEs than the amount of purified BChE or AChE we added. These results showed that sensors with combinations of multiple enzymes, cholinesterases and OP hydrolases from various sources, can be co-immobilized without reducing the activity of the enzymes when immobilized alone.

RESULTS

Comparison of soluble and immobilized enzymes: An initial rates method was used to determine the kinetic parameters for immobilized and soluble cholinesterases, OP hydrolase, and choline oxidase. The plot of substrate concentration against choline oxidase activity indicates that the soluble and immobilized enzymes have similar kinetic parameters. Furthermore, the pH dependent activityof the soluble or immobilized choline oxidase are identical. Since the pH profiles for ChEs and choline oxidase are identical (about pH 8), the coupled reaction in the assay scheme can be simultaneous optimized for both enzymes. On the other hand, the temperature profile of immobilized cholinesterase remains the same as soluble enzyme (figure 4).

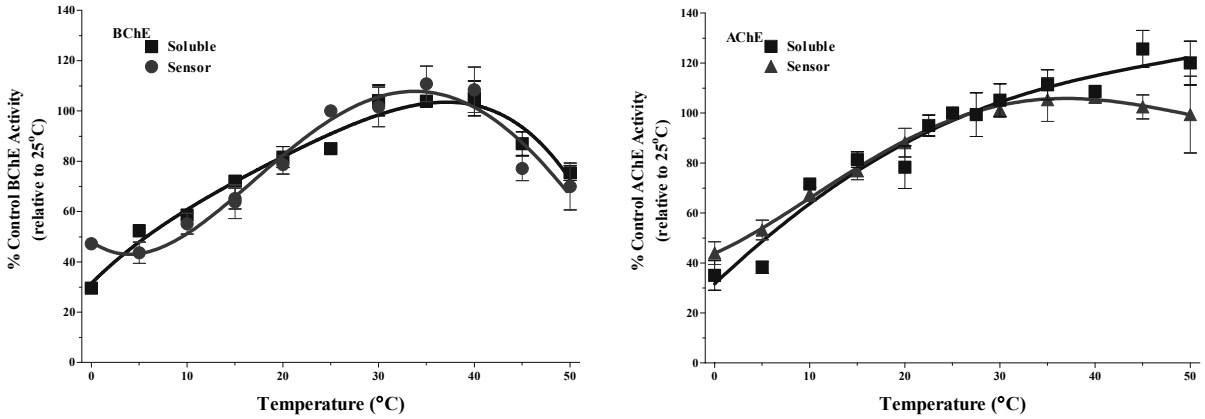

Figure 4. Temperature Dependent Activity of Soluble ChEs and Sensor ChEs (Immobilized)

Table 1. Time-Dependent Inhibition of ChEs by MEPQ

ChE	Enzyme Form	Bimolecular rate constant (M^{-1} min^{-1}) ± SD
FBS-AChE	**soluble**	**$1.59 \pm 0.52 \times 10^{8}$**
	coupled to sponge	**$1.00 \pm 0.28 \times 10^{8}$**
Equine-BChE	**soluble**	**$4.15 \pm 0.78 \times 10^{7}$**
	coupled to sponge	**$4.21 \pm 2.00 \times 10^{7}$**

Longevity of M272 ticket in aqueous environments: The M272 ticket, containing eel cholinesterase on an ion-exchange paper, lost more than 80% of its original activity in less than 5 minutes in various aqueous conditions, including, pH 8 phosphate buffer and brackish water. Therefore, the ticket, although designed for aqueous solutions, can only detect OPs in a drop or two of aqueous material placed onto the paper.

In marked contrast, the AChE activity in the immobilized sensor was stable for more than 30 days in continuous immersion in aqueous samples including fresh water, e.g., Allegheny River. Since the results are identical for autoclaved and untreated river water, the immobilized enzymes are also resistant to microbial induced proteolytic degradation. Since the properties of the immobilized enzymes for a decontamination sponge or biosensor are identical, the enzymes in a sponge won't leach to a wound or the skin. The immobilzed enzymes are also resistant to an environment of saturated organic vapors such as gasoline or diesel fumes (figure 5).

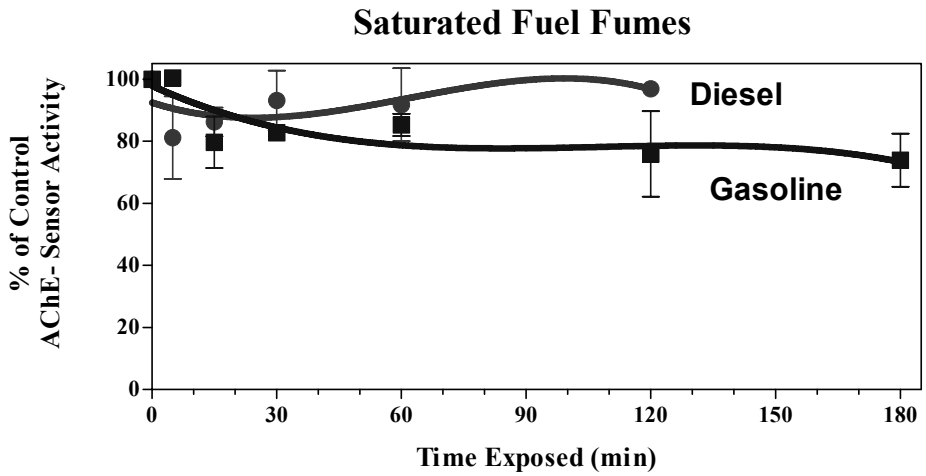

Figure 5. Sensor Exposure to Fuel Fumes (Saturated).

Sensor color reaction: Figure 6 is an example of the sensor (top), the sensor exposed to aqueous solution in the absence of organophosphate (middle), and the sensor poisoned by organophosphate (bottom). The right panel shows a standard M272 ticket where the non-immobilized enzyme is lost when merely exposed to buffer (middle). A positive control (not leached active enzyme) is shown at the top, and inhibited by OP is at the bottom. Note that for the M272 ticket, a false positive indication is observed for the middle ticket. Various color reactions are available, e.g., choline oxidase coupled reaction with amplex red reagent yielding the red chromogen resorufin for a visual indication of enzyme activity. In addition, resorufin is a fluorescent product providing increased sensitivity that could be used in a hand-held unit. Choline oxidase is one of the most efficiently immobilized enzymes we have evaluated, and is also a sensitive indicator of mustard (figure 2).

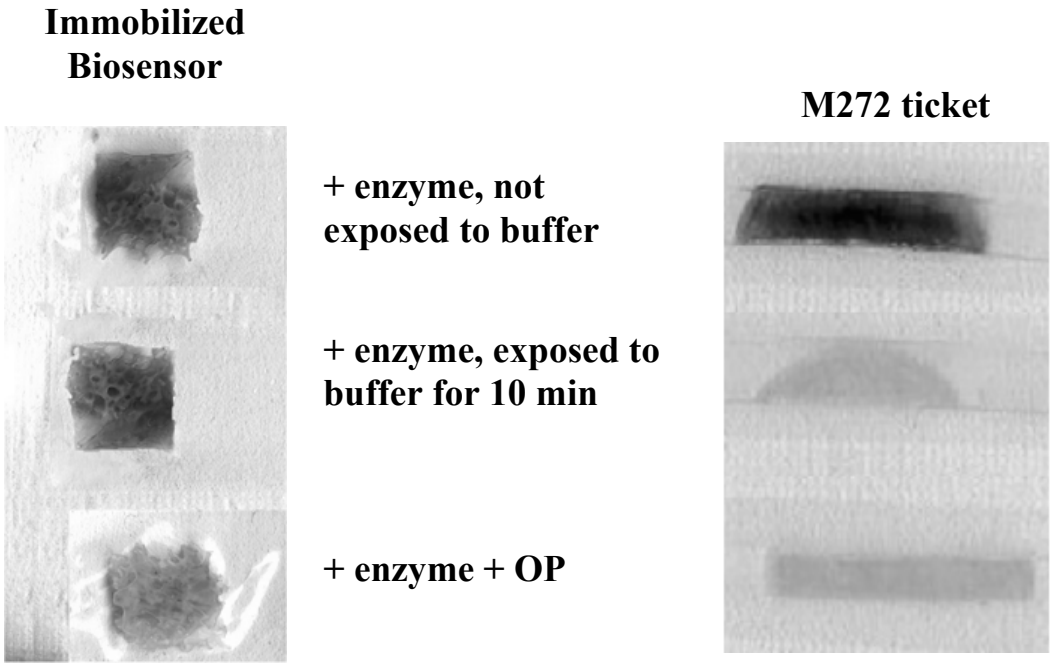

Figure 6. Comparison of Biosensor and Standard Ticket.

Sensor Sensitivity to Organophosphate: The immobilized enzyme and the soluble enzyme exhibit the same titration curve to organophosphate. Furthermore, time-dependent inhibition of AChE and BChE by the organophosphate MEPQ yielded the same bimolecular rate constants of inhibition for soluble or immobilized AChE or BChE (table 1). These data demonstrate that the immobilized ChEs detect OP in the same manner as soluble ChEs.

Table 2. Potential multiple immobilized enzymes in an immobilized biosensor

Enzyme Type	Distinguishing Characteristics
AChE, BChE	Inhibited by OPs
Laccase	Hydrolyzes VX preferentially with mediator
OPH Human serum	Hydrolyses tabun, VX poorly
Rabbit serum	Hydrolyses sarin preferentially
Pseudomonas	Hydrolyses G agents
Alteromonas undi (OPAA)	Hydrolyses soman preferentially
Squid	Hydrolyses tabun, VX poorly

The immobilized biosensor composed of enzymes that differentially hydrolyze chemical warfare agents can be construction for field determination of the type of organophosphate present, which can aid in treatment and tracking the origins of the exposure (table 2). This can be accomplished, as shown in the differential hydrolysis of OP chart, by first exposing the potentially contaminated solution to one of several discriminating (hydrolyzing) enzymes, then followed by exposure to a ChE biosensor. In the example shown in figure 7, if the solution is first exposed to OPAA and then ChE is not inhibited, then the likely OP is soman while if the solution if first exposed to laccase, and then ChE is not inhibited, the agent is most likely VX (figure 7, left and right, respectively).

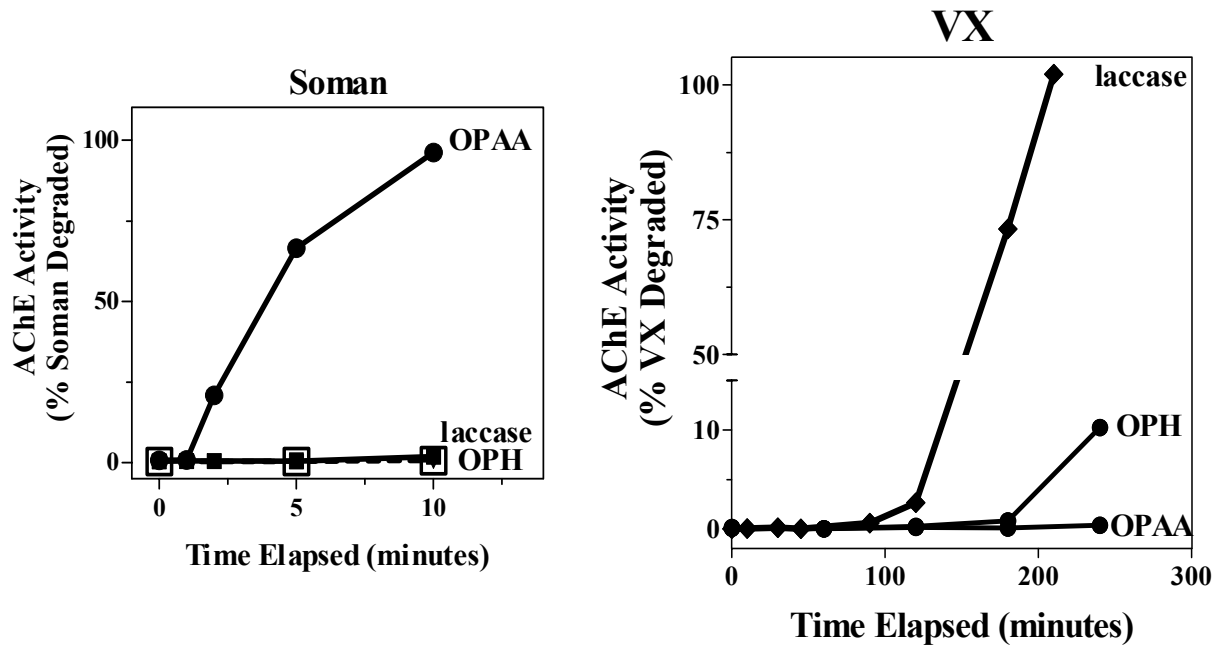

Figure 7. Immobilized Biosensor Demonstrating Differential Hydrolysis of OPs.

CONCLUSION

Due to the structural integrity of both the immobilized enzyme and matrix, the biosensor can be exposed for an extended period, and then brought to a laboratory for post-exposure identification of OP bound to the ChE-Biosensor by fluoride treatment and analysis my MS (scheme illustrated in figure 8, Polhuijs et al., 1997).

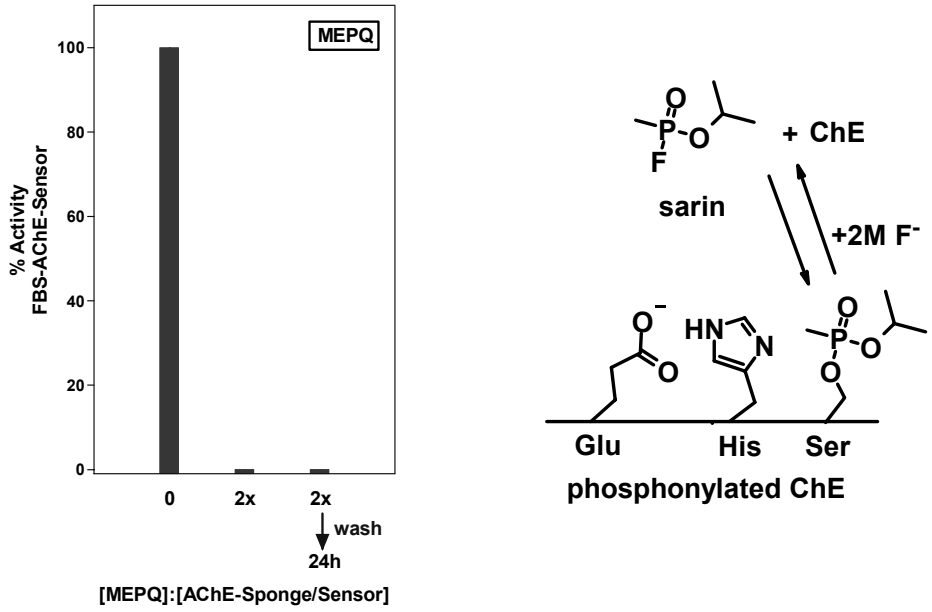

Figure 8. Post-exposure Identification of OP in the Biosensor (with fluoride ion).

We have demonstrated the rapid *in-situ* co-polymerization of ChEs at room temperature. The ChE-biosensors exhibit high activity and stability, making them suitable for a wide variety of detection tasks. The immobilized polyurethane enzymes make versatile biosensors for detecting organophosphates. These badges, by virtue of their high capacity for enzymes, stability, sensitivity, and resistance to harsh environmental conditions, can be used under diverse conditions encountered by troops in the field.

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